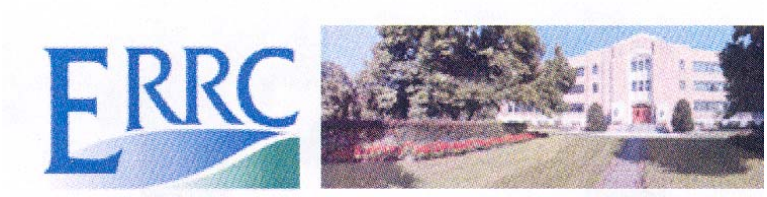

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ANTIBIOTICS

Practical Screening Procedure for Chloramphenicol in Milk at Low Parts per Billion Level

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A relatively simple screening procedure for the detection of chloramphenicol in cow's milk is detailed. The drug in 50 mL skim milk is adsorbed onto Chromosorb 102 and subsequently eluted; interfering impurities are removed by passing the effluent directly over one column containing small beds of alumina and cation exchange resin in the H^+ form. After solvent is removed, the nitro group of the drug is reduced with zinc dust in HCl, and the drug is detected by diazotization and coupling to *N*-1-(naphthyl)ethylenediamine. Milk containing ≥4 ppb chloramphenicol can be detected. A number of antibiotics and sulfa drugs permitted for use with dairy cows do not interfere with chloramphenicol detection, nor do some naturally occurring aromatic amino compounds. Nitromide (3,5-dinitrobenzamide) will interfere. It is estimated that approximately 50 samples of skimmed milk can be screened by one person during the working day. Cows secreted the drug into their milk for approximately 3 days following injection of chloramphenicol either intramuscularly or via infusion into the udder.

Chloramphenicol (CAP) is an important broad spectrum antibiotic that was first isolated from natural sources independently and almost simultaneously by Ehrlich et al. (1) and by Gottlieb et al. (2). In agriculture in the United States, its use is restricted to nonfood-producing animals because it has been reported (3, 4) to cause serious side effects in susceptible individuals and no appropriate withdrawal periods have been established (5). Nevertheless, it has long been suspected by regulatory agencies that CAP is being used surreptitiously for the treatment of mastitis in cows, because it is highly effective against that condition. The extent of its misuse, however, is not known. Although there are sensitive instrumental methods for the detection of CAP in milk (6, 7) and many methods for determining the drug clinically and in pharmaceuticals (8, 9), none of the methods appears to be adaptable to the routine analysis of a large number of milk samples. In an effort to remedy this situation, we developed a simple and sensitive qualitative screening procedure for CAP in cow's milk. The method has good specificity, does not require instruments, and can be conducted on a relatively large number of samples during the working day.

METHOD

Apparatus and Reagents

Distilled water was used throughout. Reagents were stored at room temperature unless specified otherwise.

(a) *Adsorbent.*—Chromosorb 102, 80–100 mesh (Sigma Chemical Co., St. Louis, MO). Chromosorb 102 is a styrene-divinylbenzene polymer screened from XAD-2.

(b) *Basic alumina.*—Activity grade I (Fisher Scientific Co., King of Prussia, PA).

(c) *Cation exchange resin.*—AG-50 WX8, 100–200 mesh (Bio-Rad Laboratories, Richmond, CA).

(d) *Zinc dust.*—(Aldrich Chemical Co., Milwaukee, WI). Add 150 mg to 10 mL water. Suspend by shaking just before dispensing.

(e) *HCl.*—1N.

(f) *Sodium nitrite.*—0.12% in water.

(g) *Ammonium sulfamate.*—0.8% in water.

(h) *N-1-(Naphthyl)ethylenediamine dihydrochloride (NED).*—(Sigma). 0.8% in water containing 0.1% ethylenediaminetetraacetic acid (EDTA).

Reagents (d–h) were stored in and dispensed from drop dispenser bottles (Nalge 2411 Series, A. H. Thomas Co., Philadelphia, PA). All were usable for at least 2 months. Solutions (f, g, h) were kept at 4°C when not in use.

(i) *Methanol and acetone.*—Glass-distilled (Burdick & Jackson Laboratories, Muskegon, MI).

(j) *Sea sand.*—Fisher.

(k) *Large volume Pasteur pipets.*—14.5 × 0.9 cm (Fisher, Cat. No. 13-678-8).

(l) *Shell vials.*—½ dram (1.9 mL).

(m) *Fine glass wool.*—Fisher.

(n) *Glass beads.*—4 mm (Fisher Cat. No. 11-312).

(o) *Funnels.*—Nalgene polypropylene, 2¾ in. (Macalister Bicknell Co., Millville, NJ, Cat. No. 4256-0234).

(p) *Thin layer plates (optional).*—Silica gel G without fluorescent indicator, coated on microscope slide plates (2.5 × 10 cm) (Analtech, Inc., Newark, DE, Cat. No. 01521).

(q) *Jars (for thin layer plates).*—11¼ cm high × 4.7 cm wide with foil-lined screw caps.

Scope of Method

CAP present in skim milk is adsorbed on Chromosorb 102 and potentially interfering compounds are removed after elution by passing the eluate directly through small beds of alumina and cation exchange resin. CAP is then reduced with Zn dust in HCl, diazotized, and coupled with NED to give a pink-violet color.

Preparation of Milk

When raw whole milk was involved, it was cooled to 4°C and centrifuged at 5000 rpm for 15 min. Skim milk was removed by pipet for spiking or direct screening. Pasteurized skim milk for spiking was purchased locally. Raw whole milk could not be analyzed directly because flow rates became inordinately slow.

Cleanup of Cation Exchanger

Place 10 g AG-50W in a 60 mL coarse sintered glass funnel and wash, in order, with 150 mL 1N NaOH using gravity flow, water until neutral, 100 mL 1N HCl, and water until neutral. Remove excess water by vacuum or pressure application, transfer resin to glass bottle, and add 50 mL 95% ethanol and a magnetic stirring bar.

Preparation of Chromosorb 102 Column

In a large volume Pasteur pipet, place a wad of glass wool (0.25–0.30 g) and tamp tightly and evenly. Top of glass wool bed should be above tapered portion of pipet (i.e., ca 5 mm into barrel portion). Add 300–310 mg Chromosorb 102 and a small wad of glass wool and tamp lightly. Fill pipet with water and force through with either vacuum or pressure application. Fill pipet with acetone and force through. Fill pipet again

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with acetone and let drain by gravity. Fill pipet 3 times with water and let drain by gravity. Columns can be used repeatedly.

Preparation of Cation Exchanger–Alumina Column

In large volume pipet, place, in order, 4 mm glass bead, ca 300 mg sand, 1 mL of magnetically stirred suspension of cation exchanger, and after 95% ethanol has drained, ca 1 g alumina.

Isolation of CAP on Chromosorb 102

Attach funnel to Chromosorb 102 column, using ca 2 cm Tygon tubing (5/16 in. id). Pour 50 mL skim milk (slowly at first until pipet fills) into funnel and let column effluent go to waste. When last of milk has drained through bed (25–35 min), remove funnel and tubing, and fill pipet 3 times with water, allowing complete draining of each wash. Apply vacuum or pressure after last wash until no drops of water are removed from column. Place Chromosorb 102 column piggyback in cation exchanger–alumina column (Figure 1), add 3 mL methanol to elute CAP and other adsorbed compounds, and begin collecting effluent in shell vial. Remove upper column and add 0.5 mL methanol down the sides of bottom column. Evaporate solvent under stream of nitrogen on hot plate or steam bath and remove vials as soon as possible after they have become dry.

Reduction of CAP and Colorimetric Detection

Add 1 drop of 1N HCl and 1 drop of Zn suspension to vial and rotate to wet lower sides. Let reaction mixture stand 15 min, then add 1 drop of sodium nitrite solution so that drop does not hit sides of vial. Shake and let stand 2 min. Add 1 drop of ammonium sulfamate solution, shake, and let stand 1 min. Add 1 drop of NED solution, shake, and set vial aside in the dark for 5–10 min. Hold vial up against white background. A pink, lavender, or violet color, depending on concentration, indicates presence of CAP. A control skim milk (milk from a cow not given drugs, or pasteurized market skim milk) must be run initially. Control vial can be kept frozen, and thawed for reference when needed.

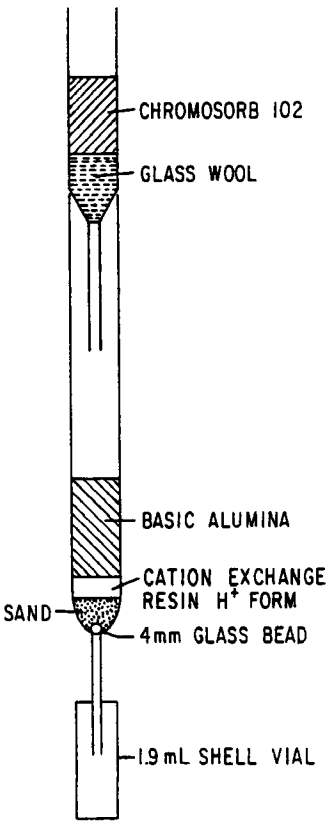

Figure 1. Set-up for purification of chloramphenicol from Chromosorb 102.

Thin Layer Chromatography (Optional)

If desired, chromatographic evidence for presence of CAP can be obtained readily to verify positive screen. Instead of running reduction step on the residue, add ca 1.5 mL *n*-hexane to the vial, heat to boiling, cool, and discard hexane extract. Let any hexane remaining in vial evaporate. Add 50 μL ethyl acetate and spot 25 μL in small aliquots ca 2 cm from the bottom of TLC plate. Spot ca 100 ng CAP on an adjacent spot and place plate in jar containing 5–10 mL ethyl acetate so that solvent does not touch plate (i.e., prop jar at ca 45° angle) and let the plate equilibrate 10–15 min. Set jar upright and let solvent ascend to top of layer (ca 7 min). Dry plate at 100°C for 5 min or at room temperature until odor of ethyl acetate is absent. Reveal spots as follows: Place plate for 2 min in jar containing solid calcium hypochlorite spread over bottom. Transfer plate to jar containing 37% formaldehyde solution for 45 s. Spray plate lightly with aqueous solution containing 1% potato starch and 1% potassium iodide. CAP can be seen as blue-black spot if present on plate at ≥60 ng (10).

If spot corresponding to CAP is seen, CAP identity can be confirmed by using the other 25 μL ethyl acetate solution. Evaporate ethyl acetate, add 2 drops of 2N HCl, cover vial with small marble, and set in simmering water bath 25 min. Evaporate acid under nitrogen stream and dissolve residue in 50 μL ethyl acetate. Spot all or part and treat as described above. Absence of spot at CAP position is further evidence that unhydrolyzed spot is CAP.

In Vivo Studies

Milk was collected from 2 Holstein cows known to be free of drugs. One cow was then injected intramuscularly with 6.25 g CAP. The other cow was given 2.5 g infusions of CAP into each quarter of the udder. Animals were milked 6 h after injection and every 12 h subsequently, up to and including 78 h.

Results and Discussion

Spiking

Approximately 20 samples of skim milk, obtained at local stores and spiked with ≥4 ppb freshly prepared CAP in water, were examined. In every case, it was possible to detect the samples containing 4 ppb CAP when compared with control samples. We were, however, unable to differentiate milk fortified with 3 ppb from the controls with any degree of certainty. Skim milk obtained from raw whole milk fortified with 4 ppb CAP before separation into skim milk and cream also was amenable to detection, suggesting that little, if any, CAP associates with the fat globules when introduced in vitro. Homogenized whole milk spiked with CAP could also be screened successfully. Skim milk spiked with 4 ppb CAP and held in either glass or plastic containers for 72 h at 4°C screened as positive; no visual difference was observed from skim milk spiked just before analysis. This suggests that if there is any in vitro binding of CAP to milk proteins, it occurs rapidly.

In Vivo Studies

Application of the screening procedure to the milk of cows injected intramuscularly with CAP showed that the drug could

be detected in the milk of the animal even at 78 h following injection, but only small amounts (about 7 ppb) were estimated to be present. When the cow received CAP via infusion into the teat canal, the drug could be detected in the milk up to 66 h later, but not at 78 h. Relatively high concentrations of CAP (deep purple test) were present in the milk of both cows at 18 h after treatment; then the concentration dropped precipitously and gradually tailed off.

Specificity

A number of drugs permitted for use with dairy cows (11) were put through the procedure with and without 4 ppb CAP to determine whether they would interfere in the screening test. All drugs were dissolved in water at a concentration of 1 μg/μL (except furaltadone, 0.5 μg/μL) and 10 μL was added to 50 mL skim milk. The drugs were novobiocin, dihydrostreptomycin, neomycin, chlortetracycline, penicillin G, sulfadimethoxine, sulfaethoxypyridazine, salicyclic acid, furaltadone, erythromycin, ampicillin, and nitromide (3,5-dinitrobenzamide). In addition, *p*-aminobenzoic acid, anthranilic acid (*o*-aminobenzoic acid), and *o*-aminoacetophenone were also tested at the same level. With the exception of nitromide, which gave a strong color, none of the compounds interfered in the screen. It is expected that other nitro compounds without a functional group amenable to removal by the alumina and/or cation exchange resin will also interfere. D-(–) *Threo*-2-amino-1-(*p*-nitrophenyl)-1,3-propanediol, a hydrolysis product of CAP, was extracted onto Chromosorb 102 from milk along with CAP, but was removed by passing the methanol eluate through alumina. It was also removed, as expected, by the cation exchange resin.

None of the drugs, except the sulfa drugs, contain an aromatic primary amino group and, theoretically, cannot interfere in the color reaction. Nevertheless, they were included in the study to determine whether they could displace CAP from Chromosorb 102 or bind it, and thus give a false negative test. Both sulfa drugs, *p*-aminobenzoic acid, anthranilic acid, and *o*-aminoacetophenone were all adsorbed from milk onto the Chromosorb 102 but were removed on one or the other traps.

Unspiked milk gives a pale straw color with the slightest hint of pink in it, and thus a control must be compared with milks containing 4 or 5 ppb CAP. Colors obtained from milk containing >5 ppb are self-evident. The color obtained from control milk is not due to the presence of a naturally occurring aromatic nitro compound because it is also obtained before reduction. This suggests that the color may be due to a very weak aromatic primary amino-containing compound incapable of exchange on the cation exchange resin, or else to the elaboration of the aromatic amino compound by acid hydrolysis during the diazotization step.

Establishment of Reaction Conditions

A number of variables were studied in an attempt to gain the greatest sensitivity while still meeting our arbitrary requirements for a practical screening test, e.g., speed, simplicity, and economy. The diazotization and coupling of the pure amino analog of CAP prepared as descsribed by Nielsen et al. (12) proceeded best in a model system when the acid strength was between 0.5 and 1.0N. In the presence of Zn dust in the reduction step, however, 0.5N HCl gave very erratic color yields when the diazotization and coupling steps were carried out. This did not occur when 1.0N HCl was used. It was concluded that the Zn reaction with HCl was sufficient to reduce the acidity of the weaker acidic medium to a point affecting the diazotization and/or coupling step(s).

The time of reduction of CAP to the amino analog by Zn dust in 1N HCl in a model system was complete in 5 min at room temperature. For the residue isolated from spiked milk, however, a 15-min reaction period was necessary for maximum color development. These reduction conditions are much milder than those used in other colorimetric (13) and fluorometric analyses (14) for CAP.

Adsorption of CAP by several polymeric resins was investigated. Besides Chromosorb 102, other resins including XAD-2, XAD-4, Duolite 861, and Duolite 863 all adsorbed the drug from milk to various degrees. Although some of the resins adsorbed more CAP than the recommended Chromosorb 102 resin, they were not selected for further study because of unfavorable flow rates and/or higher blank background color. A polymeric resin (XAD-2) was evaluated by Van Der Lee et al. (6) to isolate CAP from milk, but they reported a maximum of 20% recovery and abandoned this approach. Although we did not attempt to obtain quantitative data, it is estimated that 30–40% recoveries of CAP were obtained in our procedure.

Sample Screening

The largest number of samples that we attempted to screen at 1 time was 16. This was accomplished from start to finish in 2¼ h and used only about 2 sq. ft of laboratory space. The set-up that we used and recommend is as follows: Test tube racks (Nalgene, 72 hole, No. 5930-0013) were suspended on the rim of a polyethylene dishwashing pan (16 × 12 × 7 in. approx.). Eight Chromosorb 102 columns with funnels attached fit in each rack when properly spaced, and 3 racks will fit over the pan. After the milk had run through the columns, the resin was washed, and excess water was removed. The columns were then set piggyback in the alumina-cation exchange columns in one of the test tube racks which was raised on each end about 2½ in. above the bench by using labortory jacks or wooden blocks. The shell vials were then positioned underneath for eluate collection.

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